Effect of the microfiltration process on antioxidant activity and lipid peroxidation protection capacity of blackberry juice

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Article

Abstract: Phytochemicals are highly concentrated in berries, especially polyphenols as anthocyanins and ellagitannins. These compounds have been associated with antioxidant capacity, lipid peroxidation protection, anti-inflammatory activity, anti-carcinogenic activity, obesity prevention and others. Blackberries are commonly grown and consumed as juice in Latin-American countries. However, blackberry juice is easily fermented and different industrial techniques are being applied to enable the juice to be stored for longer periods. One important issue required for these techniques is to preserve the health-promoting capacities of blackberries. This study compared the antioxidant activity and the lipid peroxidation protector effect between a fresh blackberry juice (FJ) and a microfiltrated blackberry juice (MJ). Chemical analysis of both juices show less polyphenols concentration in the MJ. Despite this difference, values for biological activities, such as protection of lipid peroxidation, was not significantly different between FJ and MJ. These results suggest that the compounds responsible for the antioxidant activity are maintained even after microfiltration and the free radical scavenging capacity of these compounds could protect the initiation of lipid peroxidation. Microfiltration could be used as an industrial technique to produce blackberry juice that maintains biological activities of polyphenols.

Introduction

Several studies have suggested a potential health benefit of flavonoids (Beattie et al., 2005; He & Giusti, 2010). Particularly, there has been documented an inverse association between high consumption of flavonoids and the incidence of cardiovascular diseases (Mazza, 2007). Phytochemicals, such as flavonoids, can act as free radical scavengers or can up regulate endogenous antioxidant defenses. Some flavonoids suppress reactive oxygen species formation, by chelation of transition metal ions or by the suppression of superoxide-driven reactions that generate active oxygen species (Pietta, 2000).

Compared with most fruit, berries have high concentrations of a type of flavonoids known as anthocyanins (Beattie et al., 2005). Anthocyanins have been associated also with antioxidant activities, lipid peroxidation protection, anti-inflammatory activity, anticarcinogenic activity, obesity prevention and others (He & Giusti, 2010).

Blackberries are promoted as rich sources of polyphenols with antioxidant activities (Jiao & Wang, 2000; Acosta-Montoya et al., 2010). Blackberry juice consumption demonstrates the capacity to increase the plasma antioxidant capacity in humans (Hassimotto et al., 2008). Moreover, purified anthocyanin fractions of blackberry demonstrated anti-inflammatory activity by inhibition of COX-1 and COX-2 enzymes (Rossi et al., 2003; Cuevas-Rodriguez et al., 2010; Bowen-Forbes et al., 2010).

Major phenolic compounds in the blackberry variety *Rubus adenotrichus* Schltdl., Rosaceae, are anthocyanins cyanidin 3-glucoside and cyanidin 3-(6'-malonyl) glucoside and ellagitannins lambertianin C and sanguiin H-6 (Mertz et al., 2007). Both types of molecules were associated as main contributors to the antioxidant capacity in another berry *Rubus idaeus* L., Rosaceae, (Mullen et al., 2002).

Juice processing conditions cause differences in antioxidants content (Gil et al., 2000). Industrial processing with the addition of sugar and pectin; thermal treatments; and long term storage, can have marked effects on the phenolic content of berries, sometimes causing the increase of some compounds and the decrease of others (Zafrilla et al., 2001; Gancel et al., 2010).

According to Mullen et al. (2002), in raspberries these effects are more important in ellagitannins than in anthocyanins; however there is no effect in the antioxidant

capacity of the fruit. Gil et al. (2000) described that pomegranate hand press juices and commercial juices had the same anthocyanin pigments, but quantitative differences. Pomegranate anthocyanins are partially degraded or transformed into other products due to freezing procedures prior to juice extraction.

Some publications have studied changes in blackberries anthocyanins properties due to processing, storage and heat treatments. Cisse et al. (2009) explained that anthocyanins of *Rubus adenotrichus* are less sensitive to heat when compared to anthocyanins from other fruits and other varieties of blackberry. Hager et al. (2008) reported a decrease in the scavenging activity against peroxyl radicals and superoxide radicals (ORAC and PCL) after thermally processed blackberry products, but not after storage time.

Microfiltration is an application with special importance in fruit-juices. Efforts have been done to produce practical models that can be used for industrial scale and to test parameters that influence the performance of microfiltration techniques (Vaillant et al., 2008). However, it is also important to evaluate the possible effects of the microfiltration technique in beneficial roles documented for fruits-juices rich in polyphenols.

The aim of this study was to evaluate the effect of microfiltration of blackberry juice on the antioxidant capacity and the lipid peroxidation protection effect.

Materials and Methods

Sample collection

Samples were prepared with full-ripe blackberries (*Rubus adenotrichus* Schltdl., Rosaceae, cv. 'vino') harvested in different farms of the province of Cartago, Costa Rica (altitude 1864 m-2517, latitude 09º 39' 57.1"N - 09º44'40.3"N, longitude 83º53'32.1"W - 84º00'06.3"W). To prepare the fresh juice (FJ), blackberries were blended and filtrated in gauze to reduce suspended solids. The microfiltrated juice (MJ) was prepared according to Vaillant et al. (2008). Blackberries were pressed and the juice treated with a commercial enzymatic preparation (Klerzyme® 150 from DSM Food Specialties, Heerlen, Netherlands) for 1 h at 35 °C with constant agitation. The microfiltration was perform in a tubular ceramic membrane (Membralox® 1 P19-40, Pall Exekia, Bazet, France) with an average pore size diameter 0.2 μm. Both samples were stored at -30 °C until analysis.

Analytical methods

Moisture content

The moisture content was determined by the weight difference of the samples after freeze-dried. Each sample was analyzed in duplicate.

Total polyphenol content

Total phenolic content was determined with Folin-Ciocalteu assay modified by George et al. (2005). Results were expressed as milligrams of gallic acid equivalent (GAE) per gram of dry matter (DM). Each sample was analyzed in four replicates.

Anthocyanin content

Total anthocyanins content was determined by pH differential method (Lee et al., 2005b). Briefly, juice (pH 1.0 and 4.5) absorbance was measured at 510 nm and corrected with the absorbance at 700 nm. The calculation of total anthocyanin content was performed using the molecular weight (449.2 g.mol^{-1}) and molar extinction coefficient of the cyanidin 3-glucoside (26900 L.mol^{-1}.cm^{-1}). Results were expressed as mg cyanidin 3-glucoside per gram of dry matter (DM). Each sample was analyzed in four replicates.

Antioxidant capacity

DPPH radical- scavenging activity

The radical-scavenging activity (RSA) of the fresh juice and the microfiltrated juice were evaluated by assessing their direct DPPH-scavenging activity (Hyun-Jin et al., 2004). DPPH 0.25 mM was prepared in methanol and 0.5 mL of this solution incubated with 1 mL of sample dilutions. Mixtures were incubated at room temperature in the dark for 30 min and the absorbance of DPPH was measured at 517 nm. Methanol (0.5 mL) plus juice dilution (1.0 mL) were used as sample blank. Percentage of radical scavenging activity of the juice was calculated according the formula: %RSA = [1-(Abs sample/Abs control)]* 100. RSA percentage was plotted against the sample concentration and a linear regression curve was established in order to calculate the IC50, which means that amount of juice necessary to reach the 50% radical scavenging activity. Results were expressed as μg DM/mL. Each sample was analyzed in triplicate.

Oxygen radical absorbance capacity (ORAC)

ORAC assay was performed according to Ou et al. (2001). Fluorescein was used as fluorescent probe and the oxidation was induced with AAPH (2,2-azobis-2-methyl-propanimidamide-dihydrochloride). Assays were performed in spectrofluorimeter equipment (Biotek Instruments). ORAC values were expressed as Trolox equivalents (TE/g DM).

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Protection to lipid peroxidation in liposomes

Liposomes were prepared according to Pérez et al. (2003). Briefly, 25 mg of commercial lecithin was dissolved in 2.15 mL of chloroform and then 350 µL of methanol was added. This mix was dried using a nitrogen atmosphere and finally the lecithin was resuspended in 4.5 mL of warm phosphate buffered saline (PBS) and sonicated for 1 h at 4 °C to form liposomes.

To test the juice capacity to protect lipid peroxidation, an oxidative stress was induced with AAPH (2,2-azobis-2-methyl-propanimidamide-dihydrochloride). Fifty microliters of juice dilutions were mixed with 0.45 mL of liposomes and 0.2 mL of AAPH in a final concentration of 10 mM. These solutions were incubated in the dark for 2 h at 37 °C. To determine malondialdehyde (MDA) concentration, 0.2 mL of TCA 5% (trichloroacetic acid) and 1mL TBA 0.75% (thiobarbituric acid) were added and samples were heated at 100 °C for 30 min. After cooling, 0.25 mL SDS 3% was added and centrifuged at 4000 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm. MDA concentrations were plotted against the sample concentration and a linear regression curve was established, results were expressed as the amount of juice that inhibits 50% of lipid peroxidation (IC50).

Protection to lipid peroxidation in liver homogenates

Sprague-Dawley rats (220±20 g) were anesthetized and sacrificed by decapitation according to the Institutional Committee for Care and Handling of Experimental Animals of Universidad de Costa Rica (CICUA # 19-06). Liver tissue of each rat was obtained and homogenized in phosphate buffered saline (PBS) using an Ultraturrax T-25 equipment (Ika-Labortechnik) to obtain a tissue suspension at 20%. The suspension was centrifuged at 9000 x *g* during 15 min to reduce suspended solids. Seventy-five microliters of different concentrations of juices were added to 0.75 mL of liver-supernatant and incubated for 30 min at 37 °C. Subsequently, an oxidative stress was induced with TBHP (tert-butyl hydroperoxide) in the final concentration of 1.7 mM and incubated for 1 h at 37 °C. Finally, thiobarbituric acid reactive substances (TBARS) were measured as the end product of lipid peroxidation.

TBARS was assayed according to Uchiyama & Mihara (1978). Briefly, 0.25 mL of liver homogenate, 0.25 mL of 35% TCA and 0.25 mL of Tris-HCl buffer (50 mM, pH 7.4) were mixed and incubated 10 min at room temperature. Then, 0.5 mL of 0.75% TBA was added and heated at 100 °C for 45 min. After cooling, 0.5 mL of 70% TCA was added, mixed and centrifuged at 4000 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm. Concentration of TBARS was assessed using the molar absorption coefficient for malondialdehyde (MDA) of $1.56 \times 10^5 \, cm^{-1}$-$M^{-1}$ and results were expressed as nmol MDA/g liver tissue. MDA concentrations were plotted against the sample concentration and a linear regression curve was established to calculate the IC50.

The assay was performed using liver tissue from five rats. To establish basal levels, MDA levels without TBPH were assessed in each rat. Due to the color of blackberries, sample blanks were prepared in each experiment. Each juice sample was tested in triplicate.

Statistical analysis

To compare FJ and MJ results, statistical analysis was undertaken using ANOVA and Tukey's test. A *p* value <0.05 was accepted as statistically significant.

Results

Analytical methods

Table 1 shows the moisture content, total polyphenols (TP) and anthocyanins content of the fresh juice and the microfiltrated juice. The amount of total polyphenols and anthocyanins for both samples are significantly different ($p<0.001$)

Table 1. Total polyphenols and anthocyanins content in two blackberry samples.

	Moisture content *(% FW⁻¹)*	TP *mg GAE g⁻¹ DM*	Anthocyanins *mg cyanidin 3-glucoside g⁻¹ DM*
Fresh Juice (FJ)	92.0±0.1[a]	45.0±0.5[a]	11.5±0.1[a]
Microfiltrated Juice (MJ)	90.1±0.1[a]	24.9±1.0[b]	5.6±0.3[b]

DM, dry matter, TP, total polyphenols, GAE, Gallic acid equivalents. Each value is mean±SE of four replicate experiments. Means in columns followed with different letters differed significantly (p<0.001).

Table 2. Radical-scavenging activity of two blackberry samples.

	DPPH IC50 *(µg DM mL⁻¹)*	ORAC *(µmol TE g⁻¹ DM)*
Fresh Juice (FJ)	101.2±4.8[a]	547±10[a]
Microfiltrated Juice (MJ)	94.08±4.2[a]	417±11[b]

Each value is mean±SE of three replicate experiments. DM, dry matter. Means in columns followed with different letters differed significantly (*p*<0.05).

Antioxidant capacity

The DPPH and ORAC assay were utilized to evaluate the antioxidant capacity of both juices. Table 2 shows the amount of each juice necessary to reach the 50% radical scavenging activity (DPPH), and for ORAC the μmol of trolox equivalents for each sample. The comparison of values for DPPH do not show significant differences ($p>0.05$) but for ORAC the values are significantly different ($p<0.05$).

Protection to lipid peroxidation

To evaluate protection to lipid peroxidation, lecithin liposomes were incubated with both samples of blackberry juices and exposed to an oxidative stress induced with AAPH. Both juices decrease levels of lipid peroxidation measured as MDA concentration. The IC50 indicates the amount of juice necessary to decrease 50% of MDA concentration of liposomes treated with AAPH. The IC50 was 155.7±13.1 μg DM/mL for MJ and 134.7±11.0 μg DM/mL for FJ. These IC50 values do not show significant differences ($p>0.05$).

Figure 1 shows the inhibitory effect of both blackberry juices against lipid peroxidation induced by TBHP in a liver homogenates of rats. Results indicate a hepatoprotective effect against oxidative stress. FJ and MJ decrease levels of lipid peroxidation in a dose-dependent manner. The amount of FJ and MJ necessary to decrease 50% of MDA concentration of the control liver tissue treated with TBHP are 462±72 μg DM/mL and 429±118 μg DM/mL, respectively. Samples do not show significant differences ($p>0.05$).

Discussion

Microfiltration technique to produce juices involves mechanical and enzymatic treatments, transmembrane pressure and elimination of some compounds due to retained particles (Vaillant et al., 2008;

Riedl et al., 1998). All these factors affect polyphenols composition of the final juice. As it is shown in this investigation, the microfiltration process decreases the concentrations of total polyphenols and anthocyanins when it is compared to the fresh juice. Gil et al. (2000) emphasize that in the industrial process to produce juice some phenolic compounds are extracted from the fruit, but depending on the technique other ones could increase.

The antioxidant activity measured by ORAC was significantly different between FJ and MJ but is not significantly different when is measured by DPPH. According to Niki (2010) variations among antioxidant activity measured by DPPH and ORAC are expected because DPPH is based in the reaction with a stable free radical while ORAC is an assessment by a competition method. This distinction causes a different relative contribution of the compounds responsible for the antioxidant activity in each technique.

The results show that the capacity to protect lipid peroxidation is not affected by the differences in the total polyphenol composition of FJ and MJ. This was confirmed both in the liver model and in the liposomes model. It is well known that free radicals are involved to initiate lipid peroxidation (Lee et al., 2005a). Therefore, the high antioxidant capacity shown by FJ and MJ permits an effective radical scavenging and therefore protects to lipid peroxidation. Lee et al. (2005a), suggested that the ability of different compounds to scavenge ROS in liver tissue is particularly important because it's main role in metabolism of exogenous chemicals that usually generate ROS and induce oxidative stress.

In this study, despite ORAC differences for FJ and MJ, the capacity to protect against lipid peroxidation is similar for both samples, even when the lipid peroxidation is induced with AAPH or TBHP. This is in agreement with Niki (2010) who suggests that the capacity of free radical scavenging by antioxidants does not necessarily correlate with the capacity of inhibition of lipid peroxidation. Specifically, this author states that ORAC method does not show the capacity of antioxidants to inhibit lipid peroxidation.
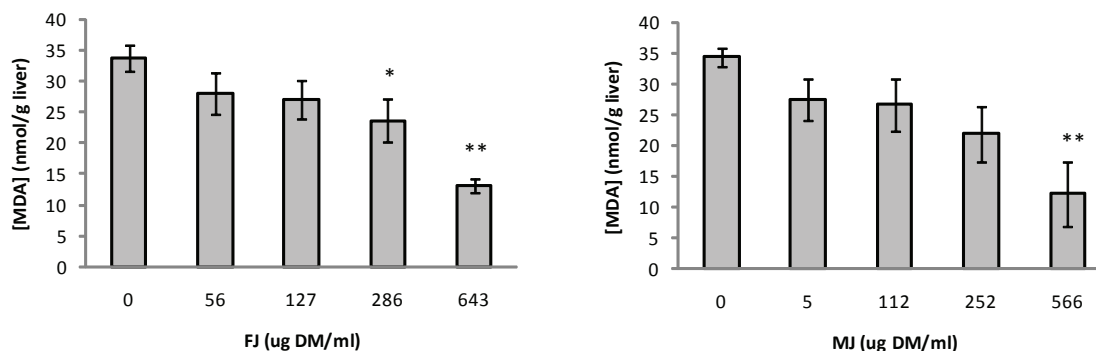

Figure 1. Protective effect of fresh juice (A) and microfiltrated juice (B) on TBHP induced oxidative stress in liver homogenates model. Each value is mean±SE (three independent experiments). *$p<0.05$, **$p<0.01$ compared with homogenates treated with TBHP.

This study concludes that differences in the amounts of polyphenols and anthocyanins after the microfiltration could alter the antioxidant capacity but do not alter the capacity to protect against lipid peroxidation. Mullen et al. (2002) evaluated changes in polyphenols quantity due to industrial process with similar results to our study. Mullen's study in raspberries evaluates the effect of short-term treatments, such as storage and freezing. For raspberries, significant differences in total phenolics were observed and changes between free and conjugated forms of anthocyanins were evident. Mullen concluded that antioxidant capacity is not due to a single anthocyanin, instead it is a combined contribution of different anthocyanins.

Blackberry juice has a very short shelf-life and that is why microfiltration process coupled to freezing storage is important to maintain the juice available for longer periods. Several other industrial processes are available, but some of them such as pasteurization present an important reduction of the health-promoting properties of processed-juice (Gancel et al., 2010). Microfiltration technique coupled to UHT packaging provides a sterile juice thanks to the pore used in the membranes. This study suggests that this procedure could permit the preservation of the capacity to inhibit lipid peroxidation in the microfiltrated juice. However, further research is needed to characterize the compounds, the interactions of polyphenols after the microfiltration process, and how these affect biological activities.

Acknowledgment

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